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Role of the Phenolic Hydroxyl Group in the Biological Activities of Simplified Analogue of Aplysiatoxin with Antiproliferative Activity

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Abstract

The 18-deoxy derivative (**3**) of a simplified analogue (**1**) of aplysiatoxin with antiproliferative activity was synthesized to examine the role of the phenolic hydroxyl group at position 18 in the biological activities of **1**. Compound **3** as well as **1** showed significant affinity for protein kinase Cδ (PKCδ), and the antiproliferative activity of **3** was slightly higher than that of **1**. However, the anti-tumor-promoting activity of **3** was less than that of **1** *in vitro*, suggesting that the phenolic hydroxyl group of **1** is necessary for the anti-tumor-promoting activity but not for the binding of PKCδ and antiproliferative activity. Moreover, PKC isozyme selectivity of **3** was similar to that of **1**, suggesting non-PKC receptors for these compounds to play some roles in the anti-tumor-promoting activity of **1**.

Protein kinase C (PKC) isozymes are serine/threonine kinases involved in cell proliferation, differentiation, and apoptosis.[1,2] The PKC family comprises at least eleven members of three distinct types: conventional (α, βI, βII, and γ), novel (δ, ε, η, and θ), and atypical (λ/ι and ζ) PKCs.[3] Naturally-occurring PKC activators such as 12-*O*-tetradecanoylphorbol 13-acetate (TPA),[4] teleocidin B-4,[5] and aplysiatoxin[6] (ATX; Fig. 1) bind to tandem C1 domains (C1A and C1B) in the regulatory region of conventional and novel PKCs. While these activators exhibit strong tumor-promoting activity,[5] bryostatin 1[7] (bryo-1; Fig. 1), a PKC activator isolated from the marine bryozoan *Bugula neritina*, shows significant anticancer and anti-tumor-promoting activities possibly through PKCδ-dependent mechanisms.[8-10] Bryo-1 has also attracted attention as a therapeutic lead for the treatment of central nervous system (CNS) diseases such as Alzheimer's disease and depression.[11] Despite its fascinating activities, however, bryo-1's low availability from natural sources and structural complexity have hampered its optimization for therapeutic use.

Several studies have indicated that the hydrophobicity of PKC activators is a critical determinant for tumor-promoting activity. For example, while hydrophobic phorbol esters such as 12-*O*-tetradecanoylphorobol 13-acetate (TPA) showed strong tumor-promoting activity,[5] less hydrophobic derivatives such as 12-deoxyphorbol 13-phenylacetate,[12] prostratin (12-deoxyphorbol 13-acetate),[13] phorbol 12,13-diacetate,[14] and PDBu[15] showed anti-tumor-promoting activity *in vivo*. Moreover, loss of the bromine atom of aplysiatoxin did not affect the affinity for PKC but reduced the tumor-promoting activity of ATX.[16] Based on these findings, we recently developed simplified analogues of ATX (**1** and **2**; Fig. 1) with less hydrophobicity and stereogenic centers, showing bryo-1-like activity.[17] Compound **1** was produced in only 22 steps, three times less than is needed to produce bryo-1, and showed antiproliferative effects against many human cancer cell lines comparable to bryo-1. Moreover, **1** showed very weak tumor-promoting activity, but significant anti-tumor-promoting activity, *in vitro*. However, its affinity for the C1B domain of PKCδ (δ-C1B) and ability to activate the PKCδ isozyme were one order of magnitude lower than

those of bryo-1.

In that study, we also suggested the geminal dimethyl group at position 6 *and/or* the phenolic hydroxyl group at position 18 of **1** to be important both for the binding of PKCδ and for the antiproliferative activity.[17] However, the exact role of the phenolic hydroxyl group in these activities remains unknown. In this communication, we report the synthesis and biological activities of the 18-deoxy analogue (**3**) of **1**.

Compound **3** was synthesized from **1** with a transfer-hydrogenation method (Scheme 1). Selective triflation of the phenolic hydroxyl group followed by transfer-hydrogenation using palladium(II) acetate, formic acid, and triethylamine gave **3**.[18] The affinity of **3** for the C1 domains of PKCδ was evaluated by inhibiting the specific binding of [^{3}H]phorbol 12,13-dibutyrate (PDBu) to the synthetic PKCδ C1 peptides (δ-C1A, δ-C1B) as described previously.[19] The affinity of **3** for these peptides was almost equal to that of **1** (Table 1) and was significantly greater than that of **2**, suggesting the geminal dimethyl group at position 6, not the phenolic hydroxyl group at position 18 of **1**, to play an important role in the binding to the C1 domains. It is worth noting that the removal of the hydrophilic phenolic hydroxyl group from the side chain of **1** did not affect the binding of PKCδ. This indicates that the side chain of these ATX analogues (**1** and **3**) is not a pharmacophore equivalent to the ester side chains of phorbol esters such as TPA that insert into phospholipid membranes. Since the introduction of oxygen or hydrophilic groups into the ester side chains of phorbol esters abolished the binding to PKC, the structure—activity data on **1** and **3** are not consistent with the pharmacophore model proposed previously based on computational methods.[20] However, the hydrophobicity of the PKC activators are important for the binding to PKC. The alkyl chain and the hydrophobic part of the benzene ring of the side chain of the analogues could contribute as the hydrophobic moiety such as the terpene moiety of teleocidin B-4 without a straight alkyl chain.

One important biological property of **1** is its antiproliferative activity. We evaluated the antiproliferative activity of **3** against a panel of 39 human cancer cell lines as described

previously.[21] The growth inhibitory activity is expressed as the concentration required to inhibit cell growth by 50% compared to an untreated control [GI_{50} (M)]. The results for the cell lines in which **1** and **3** showed GI_{50} values greater than each full panel mean-graph midpoint (MG-MID) are listed in Table 2; the MG-MID values of **1** and **3** were –4.98 and –5.09, respectively. The rest are provided as supplementary data. The antiproliferative activities of **3** were equivalent to or higher than those of **1** except for the LOX-IMVI melanoma cell lines. Notably, the GI_{50} values of **3** against St-4 and MKN45 stomach cancer cells were below 10^{-6} M. Since the affinity of **3** for PKCδ was almost equal to that of **1**, the slight increase in the antiproliferative activity of **3** might be due to the increase in hydrophobicity associated with the ability of the molecule to permeate membranes.

As mentioned above, the hydrophobicity of the PKC activators is a critical determinant for tumor-promoting activity. The tumor-promoting/anti-tumor-promoting activity of **3** was estimated by the Epstein-Barr virus early antigen (EBV-EA) induction test using Raji cell (EBV genome-carrying lymphoblastoid cell) as described previously.[23]

As shown in Fig. 2, the ability to induce EA of each compound at 10^{-7} M was measured since potent tumor promoters such as TPA and teleocidin B-4 induced maximal EA induction (*ca*. 30%) at 10^{-7} ~ 10^{-8} M.[24,25] Quite similar induction was observed at 10^{-6} M (supplementary data). TPA and ATX showed strong (28.8 and 18.4%) EA-inducing ability at 10^{-7} M, while bryo-1 with little tumor-promoting activity at the same concentration showed weak induction (1.4%) and suppressed the EA-induction by TPA significantly (6.8%). In the case of the simplified ATX analogues, **3** showed weak EA-inducing ability (9.7%), which was equivalent to that of **1** (9.4%). However, the ability of **3** to suppress the EA-induction by TPA (15.2%) was lower than that of **1** (10.1%). These results suggest that the phenolic hydroxyl group at position 18 of **1** plays an important role in the anti-tumor-promoting ability rather than in the antiproliferative activity *in vitro*.

Not only PKCδ but also other PKC isozymes (PKCs) play important physiological roles, e.g. Raji cells express PKCα, βII, and δ isozymes.[26,27] To investigate the mode of action of

the ATX analogues, PKC isozyme selectivity of ATX and its analogues were examined using the synthetic PKC C1 peptides. Recent investigation suggested that C1A domains in conventional PKCs and C1B domains in novel PKCs play critical roles in the activation of PKCs by the PKC activators. The affinities were, thus, measured by the C1A peptides of conventional PKCs and the C1B peptides of novel PKCs. The results are summarized in Table 1. While ATX showed little selectivity between conventional and novel PKCs like PDBu,[28] affinities of **1** – **3** for novel PKCs were about ten times higher than those for conventional PKC. Given that the affinity and the selectivity for PKC isozymes of **3** were comparable to those of **1**, these results also suggest an unknown receptor of **1** other than PKCs to be responsible for the anti-tumor-promoting ability of **1**.

In summary, we synthesized the 18-deoxy derivative (**3**) of a simplified analogue of ATX (**1**) to investigate the effect of the phenolic hydroxyl group at position 18 on the affinity for PKCδ, antiproliferative activity in 39 human cancer cell lines, and tumor-promoting/anti-tumor-promoting activity *in vitro*. Although **1** and **3** were almost the same in terms of binding to PKC isozymes including PKCδ, PKC isozyme selectivity, and antiproliferative activity, they differed significantly in the EBV-EA induction test with **3** showing less anti-tumor-promoting activity than **1**. The present results indicate that the phenolic hydroxyl group at position 18 is not involved in the antiproliferative activity of **1** *in vitro*. This means that the phenolic hydroxyl group would be available as a tunable site to develop superior analogues and molecular probes for analyzing the mechanism of the antiproliferative and anti-tumor-promoting activity of **1**. s

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Supplementary data. Supplementary data associated with this article can be found, in the online version, at doi: 10.1016/j.bmcl.xxxx.xx.xxx.

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18. Compound **3**: ^{1}H NMR (500 MHz, $CDCl_3$, 0.022 M, 296 K) δ 0.86 (3H, s), 0.98 (3H, s), 1.34-1.69 (13H, m), 2.21 (1H, t, *J* = 6.0 Hz), 2.40 (1H, dd, *J* = 13.1, 10.8 Hz), 2.49 (1H, d, *J* = 15.6 Hz), 2.53 (1H, dd, *J* = 13.1, 2.7 Hz), 2.62 (2H, t, *J* = 7.8 Hz), 2.70 (1H, dd, *J* = 16.7, 3.4 Hz), 2.80 (1H, dd, *J* = 16.7, 11.5 Hz), 3.70 (1H, quintet, *J* = 5.7 Hz), 3.77 (1H, ddd, *J* = 12.0, 5.7, 4.0 Hz), 3.88 (1H, m), 4.17 (1H, m), 5.18 (1H, m), 5.20 (1H, m), 7.15-7.26 (3H, m), 7.27-7.29 (2H, m) ppm; ^{13}C NMR (125 MHz, $CDCl_3$, 0.022 M, 297 K) δ 21.19, 24.77, 25.13, 25.93, 27.25, 31.28, 34.54, 34.72, 35.52, 35.93, 36.87, 36.98, 42.69, 63.72, 64.43, 68.77, 70.57, 71.74, 100.20, 125.52, 128.19 (2C), 128.44 (2C), 142.91, 169.60, 171.51 ppm; HR-FAB-MS *m/z* 475.2700 ($[M+H]^+$). Calcd. For $C_{27}H_{39}O_7$ 475.2696; $[\alpha]_D$ +41° (*c* = 0.218, $CHCl_3$, 22.7 ºC).
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Figure and Scheme Captions

Figure 1. Structure of bryostatin 1 (bryo-1), aplysiatoxin (ATX), and the simplified analogues of ATX (**1** - **3**).

Figure 2. EBV-EA-inducing ability of TPA, ATX, bryo-1, **1**, and **3**. Percentages of EA-positive cells are shown. Sodium *n*-butyrate (4 mM) was added to all samples to enhance the sensitivity of Raji cells. Only 0.1% EA-induction was demonstrated at 4 mM. The final concentration of dimethyl sulfoxide (DMSO) was 0.4%. Cell viability exceeded 60% in each experiment except for ATX (50%). Error bars represent standard errors of the mean (n = 3).

Scheme 1. Synthesis of **3**. Reagents and conditions: (a) *N*-phenyl triflimide, Et_3N, CH_2Cl_2 (91%); (b) $Pd(OAc)_2$, Ph_3P, Et_3N, HCO_2H, DMF (25%).

Table 1. K_i Values for the Inhibition of [^{3}H]PDBu's Binding by **1** – **3** and ATX

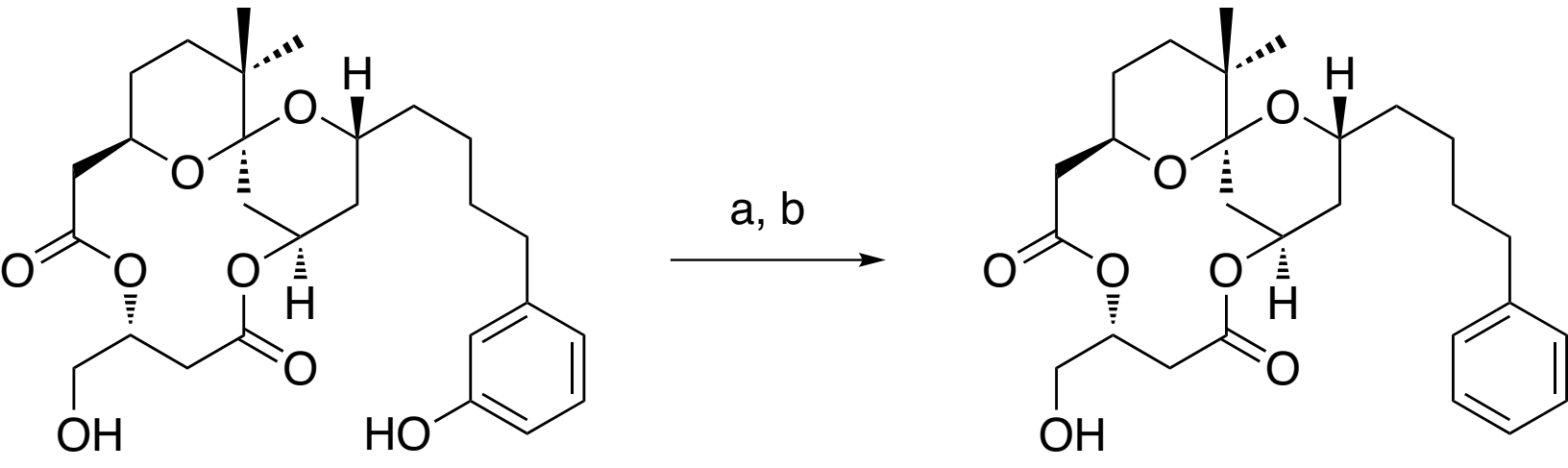
	K_i (nM)				K_d (nM)
PKC C1 peptides	**1**	**2**	**3**	ATX	PDBu[a]
α-C1A	63 (5)[b]	2,400 (100)	120 (10)	0.40 (0.05)	1.1
β-C1A	89 (5)	3,500 (400)	140 (20)	0.45 (0.08)	1.3
γ-C1A	39 (3)	1,600 (200)	80 (2)	0.63 (0.14)	1.5
δ-C1A	140[c]	6800[c]	130 (30)	12[c]	51.9
δ-C1B	7.4[c]	170[c]	9.8 (0.5)	0.41[c]	0.53
ε-C1B	25 (2)	820 (80)	37 (5)	1.3 (0.2)	0.81
η-C1B	4.4 (0.2)	180 (20)	12 (2)	0.36 (0.02)	0.45
θ-C1B	4.0 (0.5)	170 (20)	8.1 (1.0)	0.16 (0.03)	0.72

[a] Cited from ref 28. [b] Standard deviation from triplicate experiments. [b] Cited from ref 17.

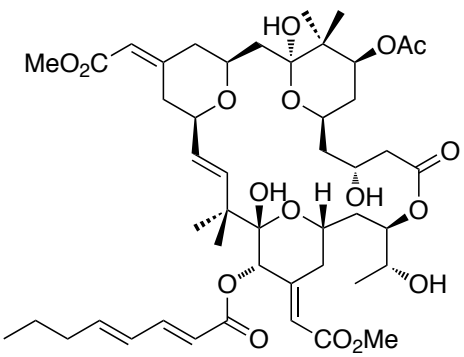
Table 2. Log GI_{50} Values for **1** and **3** against a Subset of 39 Human Cancer Cell Lines

		log GI_{50} (log M)		
cancer type	cell line	**1**[a]	**3**	bryo-1[b]
breast	HBC-4	−6.33	−6.28	NR[c]
breast	BSY-1	−4.87	−5.17	NR
breast	MDA-MB-231	−5.61	−5.67	−5.20
CNS	SF-295	−5.06	−5.14	−5.20
colon	HCC2998	−5.43	−5.53	−5.30
lung	NCI-H460	−5.60	−5.83	−5.60
lung	A549	−5.32	−5.49	−5.20
melanoma	LOX-IMVI	−5.74	−5.17	NR
stomach	St-4	−5.55	−6.05	NR
stomach	MKN45	−5.33	−6.09	NR
prostate	PC-3	−4.96	−5.26	−5.30

[a] Cited from ref 17. Antiproliferative assays of **1** and **3** were carried out simultaneously by the same method. [b] Cited from ref 22. Antiproliferative assay of bryo-1 could not carried out since the supply of bryo-1 is extremely limited. [c] Not reported.



1 **3**



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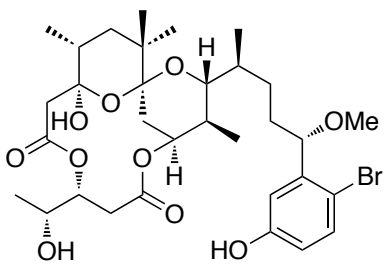
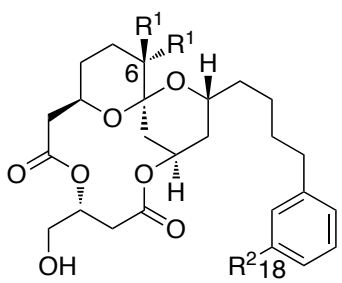


Aplysiatoxin (ATX)



1: R^1 = Me, R^2 = OH

2: R^1 = R^2 = H

3: R^1 = Me, R^2 = H